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(11) Publication number : **0 626 450 A2**

(12)

## EUROPEAN PATENT APPLICATION

(21) Application number : **94303903.2**

(51) Int. Cl.<sup>5</sup> : **C12N 15/62, C12N 15/29,  
C12N 1/21, A01H 5/00,  
C12P 21/00, C07K 15/12**

(22) Date of filing : **31.05.94**

The applicant has subsequently filed a  
Sequence Listing and declared that it includes  
no new matter

(30) Priority : **28.05.93 US 70157**

(43) Date of publication of application :  
**30.11.94 Bulletin 94/48**

(84) Designated Contracting States :  
**BE CH DE ES FR GB IE IT LI**

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(54) **Ubiquitin conjugating enzyme (E2) fusion proteins.**

(57) A novel class of fusion proteins based on the ubiquitin carrier protein, or E2, is described. The fusion proteins include, in addition to the E2 activity, a protein binding ligand having specific affinity for a target protein. It has been discovered that under cytosolic conditions, such E2 fusions will add a ubiquitin moiety to a target protein. Since ubiquitin addition triggers the endogenous cellular protein degradation pathway, such E2 fusion proteins can be used to selectively target proteins in a host for degradation. Thus, E2 fusion proteins genes can be introduced into transgenic organisms to defeat or inhibit natural activities or traits. The E2 fusion proteins can also be used by introduction into hosts for similar effects.

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Field of the Invention

The present invention relates to the field of molecular biology in general and relates, in particular, to a method for creating synthetic nucleotide sequences and resulting fusion proteins which are capable of adding ubiquitin to selected target proteins and invoking the ubiquitin-directed proteolytic process.

Background of the Invention

Living cells are constantly manufacturing proteins for metabolic as well as structural purposes within the cell. The proteins are manufactured from individual amino acids present in the cytoplasm of the cell. At the same time, each individual cell is constantly degrading excess proteins that are either present in unwanted amounts or which are no longer needed at the current stage of the cell's development. In general, proteins are constantly being degraded into the individual amino acids, which are then reconstituted separately into different proteins useful for current cellular needs.

While the mechanisms for protein synthesis are widely understood and studied, the mechanisms for protein degradation are not so well characterized. One system in which a significant amount of information has been developed is the ubiquitin-directed proteolytic pathway. This system is illustrated in Figure 1 and will briefly be described here.

Referring again to Figure 1, beginning on the right hand side, and indicated at 12, are a plurality of ubiquitin proteins. Ubiquitin is a highly conserved 76-amino acid protein found in all eukaryotes. The conservation of the ubiquitin sequence is so tightly conserved that there are only three amino acids which are varied amongst all plant and animal ubiquitin proteins yet studied. The actual three dimensional shape of the native ubiquitin molecule thus resembles the shape illustrated in Figure 1, i.e., a ball with a string or tail, composed of the carboxyl terminal 5 amino acids, is attached thereto. In the ubiquitin-directed metabolic pathway, ubiquitin is covalently attached to proteins to be degraded to "tag" them for proteolysis. This process begins when free ubiquitin in the cell associates with a protein known as the E1 or ubiquitin activating enzyme. AMP is attached to the carboxyl terminus of the ubiquitin protein in an energy consumptive reaction and then, as indicated at 14, the carboxyl terminus of ubiquitin is attached through a thiol ester linkage to a cysteine group on the E1 molecule. Then a second enzymatic protein associated with the cascade, E2, also known both as ubiquitin conjugating enzyme (UBC) or ubiquitin carrier protein, interacts with the E1 molecule and the ubiquitin moiety is transferred from the E1 protein to the E2 protein, where it is again attached through a thiol ester linkage to a unique cysteine within the E2. This reaction is indicated at 16.

Eukaryotic cells have a significant number of different E2 proteins. Each of the E2s has a core region or body portion and the core regions of the various E2s are highly conserved. Many of the E2 proteins also contain a carboxyl terminus tail portion, and there is a high degree of sequence variation among the tail portions of the E2 proteins. In the plant *Arabidopsis thaliana*, at least 15 distinct E2 proteins have been identified and there may be 30 or more. It appears that each of the isoforms of E2's are specific to specific classes of proteins. For example, one particular E2 is known to have specificity for histones. Some E2s bind specifically to free ubiquitin. Some E2s are capable of acting independently, while others require an additional enzyme, referred to as an E3, or ubiquitin-protein ligase, in order to perform their function.

The function of the E2 molecules, or the ubiquitin conjugating enzymes, is to recognize and tag proteins which will be marked for degradation. The E2 molecules perform this tag by interacting, through a mechanism not yet fully understood, with the targeted protein to be degraded, and by covalently bonding the ubiquitin protein to the protein to be degraded. In Figure 1, in the left center thereof, and indicated at 18, is the target protein. In the reaction indicated at 20, the E2 transfers the ubiquitin moiety or lysine residue into the target protein. The target protein is illustrated again in the center of the figure with several ubiquitins attached. In cells *in vivo*, many repeats of ubiquitin are similarly added to the protein to be degraded. This can occur either because a chain of ubiquitins is added in one step to the protein to be degraded or it can occur where a single ubiquitin is added to the protein to be degraded, after which the chain of ubiquitins are added in sequence to that ubiquitin. Some proteins with only a single ubiquitin attached may survive in an altered form, but the research to date indicates that all proteins tagged with a multi-ubiquitin tag are degraded promptly.

In any event, proteins which have the ubiquitin repeat units attached to them are recognized by a particle existing *in vivo* in eukaryotic cells known as a proteasome, indicated at 22 in Fig. 1. The proteasome is a complex of proteolytic enzymes which recognizes proteins by the ubiquitin "tag" and which then proceeds to quickly and efficiently degrade the marked protein into its constituents amino acids. In this same process, the ubiquitin is released from the protein and is recycled. The amino acids thus produced are released into the cytoplasm. No presently existing tool of molecular biology permits the targeted degradation of native proteins expressed in living organisms. The closest tool of molecular biology analogous to this technique is the evolving

technology of antisense. In antisense technology, a gene is introduced into a living cell which is designed to produce an antisense RNA transcript. The antisense RNA transcript is intended to hybridize under *in vivo* conditions with an mRNA transcript natively present in the cells. The hybridization of those two RNA molecules creates a double stranded complex which is then degraded by yet uncharacterized cellular mechanisms. The net result is that the level of expression of the gene creating the target RNA is dramatically reduced or, in some cases, practically eliminated. It has also been proposed that free antisense RNA molecules might be delivered into the blood stream of vertebrates in order to suppress the expression of unwanted proteins.

There are no known prior attempts to use the ubiquitin protein degradation pathway to artificially induce ubiquitination and degradation of targeted proteins.

### Summary of the Invention

The present invention is summarized in that synthetic E2 molecules are created which include a body portion, a tail portion which is either natural or synthetic, and a heterologous protein binding ligand attached to the carboxyl end of the tail portion. It has been demonstrated that such molecules are capable of adding a ubiquitin particle to target proteins recognized by the protein binding ligand.

It is a further object of the present invention to describe gene sequences which are capable of causing the expression of such synthetic E2 protein recognition molecules in heterologous hosts.

It is also a feature of the present invention in that indigenous proteins in transgenic organisms can be targeted for degradation through the use of such synthetic E2 protein recognition molecules.

Other objects, advantages, and features of the present invention will become apparent from the following specification when taken in conjunction with the accompanying drawings and sequence listings.

### Brief Description of the Drawings

Fig. 1 is a schematic illustration of the ubiquitin-directed protein degradation cycle as it occurs in eukaryotic cells.

Fig. 2 is a schematic illustration of a prototype molecule constructed in accordance with the present invention.

Fig. 3 is a schematic illustration of a synthetic molecule constructed of UBC4 and a c-myc ligand, and its use to tag a target antibody molecule with ubiquitin.

Fig. 4 is a schematic illustration of a molecule composed of UBC4 and TGF $\alpha$  protein, and its use in directing ubiquitin addition to a target protein.

Fig. 5 is a schematic illustration of a molecule composed of UBC4 and gene V, and its use in directing ubiquitin addition to a target protein.

Fig. 6 is an illustration of a molecule composed of UBC4 and protein A and its use in directing ubiquitin addition to a target protein.

Fig. 7 is a schematic illustration of a proposed molecule consisting of UBC4 and an antibody, and its use in directing ubiquitin attachment to a protein.

### Description of the Preferred Embodiment

It is proposed, in accordance with the present invention, that a novel class of E2 derived fusion proteins be constructed. This novel class of E2 fusion proteins is illustrated, as a prototype, in Fig. 2. This class of E2 proteins, are constructed so as to add a ubiquitin moiety with specificity to a target protein, and are composed of three main constituents as illustrated in Fig. 2. One constituent, designated at 24, is a natural or artificial E2 protein core region. The second element, indicated at 26, is a spacer which can be either an artificially constructed spacer or a tail region from a native E2 isoform. The third constituent, designated at 28 in Fig. 2, is a protein binding ligand, again either natural or artificial, which has unique specificity to a target protein of interest.

The function of the E2 fusion protein molecule described above, is to add one or more ubiquitin residues to a target protein of interest, such a protein being indicated at 30 in Fig. 2. Since the pathway of ubiquitin-directed protein degradation *in vivo* in cells is actuated by the addition of ubiquitin to proteins to be degraded, all indications are that the addition of such ubiquitin protein tags to specific proteins will cause their degradation. In this manner, it becomes possible to target specific proteins for degradation through the use of novel E2 proteins such as those described above.

As also described briefly above, each of the class of proteins called ubiquitin conjugating enzymes, or E2s, consists of one or both of two main constituent domains. All known E2 isoforms include a core region, which

is usually approximately 150 amino acids in length. The core region includes within it a unique cysteine residue, which is necessary for the formation of the ubiquitin-E2 thiol ester intermediate necessary for the cycle illustrated in Fig. 1. It is not clear that the cysteine must be unique. The core region of various E2s may include an amino terminus with a region containing hydroxylated and uncharged residues, which are associated with increasing the rate of thiol ester formation. Described below in SEQ ID NOS: 1 and 3 below are the coding regions for two different E2s isolated from two different plant species. The two sequences encode proteins (SEQ ID NOS: 2 and 4) which contain similar, though not identical, core regions. These two sequences are exemplary, and several other E2 core region sequences have been sequenced and published. What is required of the E2 core region is that it be capable of functioning so as to participate with an E1 protein linked to a ubiquitin in a transesterification process to form the thiol-ester linkage of the E2 core region to the ubiquitin. The E2 core region must also be capable of transferring the ubiquitin to the target protein in the manner described in Fig. 2 and below. It is not believed that the source of origin of the E2 protein is of any criticality. Since ubiquitin proteins are extraordinarily highly conserved among all eukaryotes, and since the chemical interaction required in the procedures described here is between the E2 molecule and the ubiquitin, the core regions from E2 molecules from virtually all eukaryotic sources should be able equivalently well *in vivo* to bind to and transfer endogenous ubiquitin. As demonstrated below as well, plant-origin E2 core regions are capable of adding ubiquitin to animal or viral origin proteins and are capable of using either plant or animal origin ubiquitins. Hence, there appears to be little or no species, or even kingdom specificity, to the origin of the E2 domains. Similarly, any eukaryote which expresses a compatible ubiquitin is a target for this approach. All known eukaryotic ubiquitins fit this criteria, and hence it appears that E2s from any eukaryotic source may be used in conjunction with ubiquitins present in any target host.

There are differences between the two eukaryotic E2 isoforms which are used in the examples below. UBC1 is an E2 isolated from *Arabidopsis thaliana*, while UBC4 is an E2 isolated from wheat. The E2s UBC1 and UBC4 differ in both their specificity and their method of activity. The native UBC4 E2 is capable, *in vitro*, of binding to the target protein alone, without the need for an ubiquitin protein ligase, or E3, molecule. UBC4 strongly associates with histones, due to an interaction between the strongly acidic carboxyl terminus tail of UBC4 which interacts with the basic histone molecules. Therefore, UBC4 may be associated with histone degradation in its native system, although this is not proven. UBC1 requires the presence of the ubiquitin protein ligase, or E3, molecule in order to direct the ubiquitin addition to a target protein. In comparing the UBC1 and UBC4 molecules, the principal difference at a structural level lies in the fact that UBC4 possesses a tail region at the carboxyl terminus of its protein sequence, while UBC1 possesses no such tail. These two examples are illustrative of the difference between E3-dependent and E3-independent E2 isoforms. It has been previously reported by the investigators here that the transfer of a UBC4 tail from UBC4 to UBC1 confers on the UBC1 protein an affinity for histones. Hence, it has been postulated that the tail portion of E2 is responsible for the affinity to the protein which is to be signaled for degradation. Separately, it is now reported here that in the absence of a spacer region between the carboxyl terminus of a tail-less E2, such as UBC1, an E2 fusion protein strategy does not result in ubiquitin addition to the substrate protein. Thus a tail-less E2 variant cannot be used in the process of the present invention without adding a native or artificial spacer or tail to its carboxyl terminus. Conversely, as described below, it has been found that the addition of a synthetic spacer region between the core region of an UBC1 type E2, and the protein-binding ligand, permits functional utilization of the UBC1 type E2 molecule in a function E2 fusion protein which operates independently of E3. Accordingly, it is a requirement of an effective E2 fusion protein molecule in accordance with the present invention that between the core region of the selected E2 and the protein-binding ligand there be a spacer. A natural E2 tail may serve as the spacer, or an artificially constructed amino acid sequence will work additionally well as the spacer. It is also possible that the spacer can be added to or a part of the protein-binding ligand. For example, many single chain antibody sequences include rigid amino terminal domain that could serve as the spacer. It has been found that the spacer should optimally be at least four amino acids long, in order to permit both the proper functioning of the E2 core region and the proper binding and specificity of the protein-binding ligand for the substrate to which ubiquitin is to be added.

Also shown in Fig. 2 as a part of the E2 fusion protein is a protein-binding ligand 28, intended to have specific affinity for a targeted protein. The protein binding ligand can be any of a large number of possible amino acid sequences, as long as the encoded ligand provides specific binding to the target protein of interest. There are many examples of possible protein binding ligands. Protein science has generally become aware of the specific affinities between protein hormones, and the membrane bound receptor proteins with which they normally interact. It is therefore possible to use either one of these proteins, either the receptor or the hormone, as a protein binding ligand for the other. The responsible binding domain of either the hormone or the receptor may also be used, rather than the entire protein. It is possible, as described below, to use protein A as a ligand which binds specifically to antibodies. Also illustrated below is the use of a single protein which, in its native

form, forms a dimer with other similar proteins in vivo, to target ubiquitin addition to the associated proteins in vivo. This demonstrates that native protein-to-protein associations may be used to identify protein-binding ligands. It is possible to use as the protein binding ligand an epitope which will be specifically bound by a particular antibody. In most situations, however, what will be ultimately desired for the protein binding ligand would be an antibody, or at least the recognition portion of an antibody, such as a single chain fragment antibody. For proteins which are not known to have another protein having specific attraction for them, the ability to create and utilize antibodies recognition domains specific for the target protein is essential. Through the use of antibody recognition sequences for the protein binding ligand, it is possible to target virtually any protein in a biological system for ubiquitin addition, and ubiquitin-directed protein degradation, in the manner described herein using an E2 fusion protein. All that seems to be required is for the target protein to have at least one lysine residue to which the ubiquitin may be attached.

It is possible to produce the E2 fusion protein of Fig. 2 by a variety of processes for use in ubiquitin-directed protein degradation. For example, it is possible to clone and/or construct a DNA sequence which, in its entirety, codes for an E2 fusion molecule of the class shown in Fig. 2. The DNA coding sequence could include, working 5' to 3', first the coding sequence for a selected E2 core region, such as those native sequences described in the sequences below, or could include a consensus or artificial E2 core region sequence. The DNA coding sequence would then include 3' to the core region sequence, a sequence encoding a spacer, or a tail region from E2. Again, this portion could be native or artificial, and both the core region and spacer could be from a single native E2, as in the case of UBC4 below. Finally, at its 3' end, the coding sequence would include the coding region for the protein binding ligand. When such a coding region for an E2 fusion protein is placed behind a promoter effective in a host of choice, and 5' to a transcription terminator, an expression cassette for the recombinant sequence expressing an E2 fusion protein, such as illustrated in Fig. 2, is created. That expression cassette can then be transformed into a heterologous host and expressed. Various transformation techniques for plants and animals are known in the art and need not be described further here. The E2 fusion protein thus expressed will then direct ubiquitin addition to a target protein of interest in vivo. This ubiquitin tagging will direct degradation by the cell, using its normal processes, of the target protein. In this way, transgenic organisms can be created which differ from their native or non-transformed ancestors by the active degradation of a specific unwanted protein. In this way it is possible to "turn off" proteins which are not desired to be expressed. This approach can be used in animal, plant systems, microbial or fungal systems which has an active ubiquitin-directed proteolytic pathway, to turn off unwanted activities, structures, traits or activities.

Alternatively, E2 fusion proteins such as those described-and illustrated in Fig. 2 can be produced by expression in one host for ultimate delivery into another. For example, it is possible to take a synthetic coding sequence, as described in the preceding paragraph, and express that sequence in a prokaryotic host to produce E2 fusion protein, assuming only that the promoter is properly chosen. For example, it is quite common to produce therapeutic proteins by the fermentation of prokaryotic bacteria, and then to, subsequently isolate and purify the desired protein. In this fashion, useful quantities of E2 fusion proteins can be produced and isolated. The protein can then be delivered to an organism for possible therapeutic or other treatment. If production of the E2 fusion protein is performed outside of the host, it is envisioned that further treatment or modification of the E2 fusion protein may be desired. For example, liposome encapsulation of E2 fusion proteins may aid in their introduction into target cells. Alternatively, further protein domains could be added to the amino terminus of the E2 fusion proteins to target cellular receptors to induce introduction of the proteins into targeted cells in vivo.

A wide variety of possible applications for this technology are envisioned. In plant systems, it becomes possible to target the degradation of a specific enzyme, to turn off an unwanted plant metabolic pathway. For example, it now becomes possible to alter the secondary metabolite products of a plant by targeting for degradation one or more enzymes in the cascade for the unwanted secondary metabolite. The E2 fusion protein approach offers the promise of another mechanism for the control of virus infection in plants, by targeting the degradation of either the viral coat protein or one or more viral enzymes, e.g. a transcriptase, necessary for viral replication or activity in infected plant cells. It also becomes possible to target the degradation of specific cellular receptors, to diminish sensitivity to one or more otherwise undesirable effects caused by exposure to some environmental stimulus. It is possible to target the degradation of specific plant phytochromes enzymes, so as to alter plant vigor and growth.

In mammalian systems, analogous applications for this technology are also envisioned. Again, attempts can be made to interfere with viral pathogenicity by targeting for degradation either viral coat protein or enzymes necessary for viral replication or infection. It is possible to target for the degradation of onco-proteins, as a possible therapeutic strategy to try to slow or alter the process of oncogenesis. It is possible to target for degradation unwanted growth factors, or factors associated with the processes which are desired to be hindered for some period of time, such as blood clot formation. In general, it is envisioned that this technology

offers the ability generally to target specific endogenous proteins for degradation, and to thus inhibit the otherwise native expression of proteins. In this manner, this technology provides an alternative to the "antisense" technology as a mechanism for interfering with native cellular processes in a quite specific manner.

This approach does have one significant advantage over the "antisense" approach. Antisense requires RNA-level sequence identity to be effective and, hence, for an activity caused by several isozymes, each isozyme must be separately targeted. The approach here could be used to target an active domain to direct degradation of all proteins with the domain. Hence protein families can be targeted with a single E2 fusion protein as well as specific proteins.

## 10 EXAMPLES

### 1. Common Methods and Materials

#### A. Materials

15 Oligonucleotides were synthesized as described in the sequences listings below, and were provided by E.I. du Pont. Restriction enzymes, M13mp18 single stranded DNA, and T4 polynucleotide kinase were purchased from New England Biolabs. VCS-M13 was purchased from Stratagene. Shrimp alkaline phosphatase (SAP) was purchased from United States Biochemical. Tag DNA polymerase was purchased from Perkin Elmer Cetus. E1 protein was synthesized in E. coli utilizing the TaUBA1 gene as described in Hatfield and Vierstra. 20 J. Biol. Chem., 265:15813-15817 (1992), and was then purified by the method described in Ciechanover et al., J. Biol. Chem., 257:2537-2542 (1982). Human ubiquitin was used and was purified by the method of Haas and Wilkinson, Biochem. Prep., 15:49-60 (1985), and was then radiolabeled with carrier-free Na<sup>125</sup>I by the chloramine-T method as described by Ciechanover et al., Proc. Natl. Acad. Sci USA, 77:1365-1368 (1980). 25 Rabbit reticulocyte extract (untreated) was purchased from Promega Corporation. The wheat germ extract was prepared according to the method of Hatfield and Vierstra, Biochem. 28:735-743 (1989). Anti(c-myc) monoclonal-antibodies derived from clone 9E10, and the c-myc peptide, were purchased separately from Oncogene Science, Inc. Alkaline phosphatase-conjugated goat anti-mouse immunoglobulins were purchased from Kirkegaard and Perry Laboratories Inc. Human epidermal cell membranes containing the epidermal growth factor receptor (EGFR) and purified epidermal growth factor (EGF) were a gift. The cDNA copy of the transforming 30 growth factor  $\alpha$  (TGF $\alpha$ ) gene was a personal gift, but is described in Derrick et al., Cell, 38:287-297 (1984). Monoclonal antibody against the ribosomal protein S3 was a gift. All other reagents were purchased from Sigma.

Chromosomal DNA from Staphylococcus aureus was purified as described for E. coli by Perbal, A Practical Guide to Molecular Clonings (1984). E. coli cell extracts infected with M13 were prepared by inoculating LB 35 media, containing tetracycline at 12  $\mu$ g/ml, with 1/100th volume of an over-night culture of the E. coli strain of XL1-Blue. The culture was incubated with vigorous shaking for 1 hour at 37°C and infected with VCS-M13 (Stratagene) at a multiplicity of infection of 10. Infected cells were incubated for an additional 1 hour, after which kanamycin was added to 100  $\mu$ g/ml, and the culture was incubated for an additional 6 hours. The cells were 40 harvested and lysed as described for the E. coli cultures following induction in Sullivan and Vierstra, J. Biochem., 266:23878-23885 (1991). The M13-uninfected cell extracts were prepared as described above except that M13 phage and kanamycin were omitted.

#### B. Construction of UBC1 and UBC4 Expression Vector Cassettes.

45 Unless stated otherwise, all techniques were performed according to the protocols as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Vols. 1-3 (1989). Creation of the expected site-directed versions and insertion of the various DNA fragments described below in their proper orientation was confirmed by subsequent sequence analysis. All studies began with the cDNA copies of the UBC4 gene from 50 wheat (Triticum vulgare) as described in Sullivan and Vierstra, J. Biol. Chem. 266:35:23878-23885 (1991), and the UBC1 gene from Arabidopsis thaliana from Sullivan and Vierstra, 1991, *supra*, contained within the phagemid, pUC118 or pBluescript from Stratagene, respectively. A unique XhoI restriction endonuclease site was placed immediately upstream of the translation termination sites in both the UBC1 and UBC4 coding regions by site-directed mutagenesis performed as described by Kunkel et al., Methods in Enzymol., 154:367-382 55 (1987). This site-directed mutagenesis was performed using the oligonucleotides RV155 and RV131 which are presented as SEQ ID NOS: 6 and 5 below respectively. The mutagenized UBC1 cDNA was then ligated into the pET 3a plasmid vector containing the UBC1 gene (Sullivan and Vierstra, 1991) as a SalI/BamHI cassette replacing the wild-type UBC1 gene. This replacement created an expression plasmid designated pET-UBC1.

The mutagenized UBC4 cDNA was ligated into the pET 3a plasmid vector containing UBC4 (Sullivan and Vierstra, 1991) as a SphI/BamHI cassette replacing the wild-type UBC4 gene in that vector. This replacement created an expression plasmid designated pET-UBC4. Insertion of the XhoI site into UBC1 and UBC4 resulted in the addition of a dipeptide, Leu-Glu, to the carboxyl terminus of the native protein sequence of each protein as encoded by the respective DNA expression plasmids.

#### C. Synthesis of UBC1 and UBC4 Proteins With Carboxyl-Terminal Additions.

Additions to the Carboxyl-terminus of UBC1 and UBC4 proteins was accomplished by ligation of appropriate synthetic oligonucleotide pairs or double stranded DNA fragments into the XhoI site at the 3' end of the corresponding DNA coding sequences for the respective proteins. A phosphate group was first added to the 5'-end of the synthetic nucleotides using T4 polynucleotide kinase. Reactions containing the oligonucleotide at a final concentration of 6.67  $\mu$ M, 400  $\mu$ M ATP, and 0.33 units/ $\mu$ l of T4 polynucleotide kinase dissolved in T4 polynucleotide kinase buffer, and were incubated at 37°C for 30 minutes. The complementary oligonucleotide pairs were annealed to each other through the addition of one-tenth volume of 10x Annealing Buffer (Biorad) and the mixture heated to 80°C. Reaction mixtures were then allowed to cool to room temperature over approximately 1.5 hours.

Prior to the insertion of the altered DNA coding sequences including carboxyl terminal additions into the expression plasmids, pET-UBC1 and pET-UBC4, the plasmids were digested with XhoI and treated with shrimp alkaline phosphatase (SAP) to reduce the frequency of self ligation. Dephosphorylation of XhoI-digested plasmids was performed using SAP as using the methodology described by the supplier. After dephosphorylation, the remaining enzyme was denatured by heating the entire reaction mixture to 70°C and holding it for 10 minutes.

#### D. Construction of c-myc expression vectors.

This example was intended to demonstrate the use of an epitope as the protein binding ligand. The epitope chosen, because of convenient access to the monoclonal antibody, was c-myc. The oligonucleotide pair, designated RV138 and RV139, presented as SEQ ID NOS: 7 and 9 respectively below, are designed to form a double stranded DNA cassette that encodes a 10-amino-acid epitope SEQ ID NO: 8 recognized by the mouse monoclonal antibody designated clone 9E10. The mouse monoclonal antibody clone 9E10 was generated against the oncogene protein c-myc as described by Evan, et. al., *Mol. Cel. Biol.* 5:12:3610-3616 (1985), and the ten amino acid epitope for the anti-c-myc antibody is described in Koledziej and Young, *Methods Enzymol.* 194:508-519 (1991). The c-myc epitope cassette was ligated into the XhoI site of pET-UBC1 and pET-UBC4 to create two plasmids then designated pET-UBC1-(c-myc) and pET-UBC4-(c-myc). The presence of the c-myc epitope on the protein expressed on each of these plasmids was established by inducing expression of the E2 proteins made by these plasmids and then screening for c-myc positive strains by immunoblot analysis using with the c-myc specific antibody. In this way, it was assured that the plasmids properly expressed the epitope recognized by that antibody.

#### E. Construction of a UBC1-spacer-(c-myc) expression vector.

In addition to constructing a vector in which the c-myc epitope was ligated to the carboxyl terminus of the UBC1 E2 protein, it was also desired to add a spacer between the core region of the UBC1 E2 molecule and the protein-binding ligand, in this case the c-myc epitope. To do this, an oligonucleotide pair designated RV136 and RV137, set forth as SEQ ID NOS: 16 and 18 below, was designed. This oligonucleotide pair, when annealed and expressed in a host, is intended to create a 10 amino acid spacer consisting of the amino acids set forth in SEQ ID NO: 17. This cassette was further designed to ligate into XhoI site of pET-UBC1. Upon ligation of the spacer oligonucleotides, the original XhoI site in pET-UBC1 was lost and a new XhoI site was created at the 3'-end of the spacer cassette to allow for further insertions. After insertion of the spacer cassette, the c-myc cassette was then ligated into the XhoI site in the spacer of pET-UBC1-spacer, creating the new expression vector plasmid designated pET-UBC1-spacer-(c-myc). This construction was intended to demonstrate the sufficiency of an artificial spacer between the core protein of the E2 and the protein binding ligand. This construct, and its use is generally illustrated in Fig. 3.

#### F. Construction of a UBC4-TGF $\alpha$ expression vector.

To obtain a sequence for a TGF $\alpha$  coding region, it was decided to clone by PCR a DNA region encoding

amino acid 41-89 of the human peptide hormone transforming growth factor  $\alpha$  (TGF $\alpha$ ). The activity of the TGF $\alpha$  hormone has been determined to result from its conjugation to a specific cellular receptor, known as the epidermal growth factor receptor (EGFR) present as a cellular receptor in many cells in a human body. The portion of the coding sequence for the TGF $\alpha$  gene (amino acid 41-89 of the native protein), includes the binding ligand which natively binds to the EGF receptor.

Shown as SEQ ID NOS: 10 and 11 below are a pair of oligonucleotides designated RV151 and RV154, which were designed to be primers for amplification of the desired coding region from TGF $\alpha$  by polymerase chain reaction (PCR). The PCR template consisted of a plasmid containing the cDNA copy of the TGF $\alpha$  pre-hormone form, the sequence of which can be found in GENE BANK accession # M31172. Additional DNA sequence was added to oligonucleotide RV151 to encode a XhoI site. The PCR was performed on the template to create multiple copies of a PCR product. The Sall site was then used to ligate the PCR product into the XhoI site of pET-UBC4 to create an in-frame coding region fusion between the coding region for UBC4 and TGF $\alpha$ . Likewise, oligonucleotide RV154 was designed to contain a stop codon at the 3'-end of the hormone coding region followed by a Sall site. The product of PCR amplification was digested with Sall and XhoI, and was then ligated into the XhoI site of pET-UBC4, to create an in-frame fusion of UBC4 and TGF $\alpha$  proteins while maintaining the XhoI site at the 5'-end thereof. This plasmid was designated pET-UBC4-TGF $\alpha$ . This fusion protein and its use is schematically illustrated in Fig. 4.

#### G. Construction of a UBC4-GENEV expression vector.

GeneV is a protein from the M13 phage. In its native form, the protein associates in homo-dimers. The intent of this expression was to test the ability of a single subunit of a dimer to serve as a protein binding ligand within the present invention. Again the protein coding sequence of interest was prepared by PCR reaction. The oligonucleotide pair, RV220 and RV221, SEQ ID NOS: 13 and 12 respectively, were designed to amplify by PCR the complete coding region of the GeneV protein from the bacteriophage M13, as set forth in GENE BANK accession # VB0018. The oligonucleotides also include unique XhoI and Sall sites such that the GeneV coding region amplified by PCR could be digested with XhoI and Sall and the resulting 271 base pair fragment then could be ligated into the XhoI site of PET-UBC4 and the same method described with the UBC-TGF $\alpha$  construct described above. Again this construct made, in a similar fashion, an in-frame fusion of the UBC4 protein with the GeneV protein domain, and the construction was designated PET-UBC4-GeneV. This construction and its use are schematically illustrated in Fig. 5.

#### H. Construction of a UBC4-ProteinA expression vector.

ProteinA from *Staphylococcus aureus* has a high natural affinity for many classes of antibodies. The antibody-binding region of ProteinA binds to the Fc portion of antibodies rather than to the antigenic recognition site. This was used to test to see if antibodies could be targeted for destruction, although this test would be generic to antibodies and not specific to the epitope recognized by the antibodies. This example is also intended to demonstrate that classes of proteins can be targeted through the use of a domain for the protein binding ligand that recognizes classes of proteins.

Again it was decided to amplify by PCR the coding region of the antibody-binding D domain (amino acids 90-193) of ProteinA, as set forth in GENE BANK Accession # M18264. A pair of oligonucleotide primers, designated RV242 and RV238, set forth as SEQ ID NOS: 14 and 15 was designed to both amplify the PCR product and add the appropriately desired restriction sites at the end of the PCR product. The PCR process was performed on *S. aureus* chromosomal DNA. Again, the oligonucleotides provided for Unique XhoI sites at each ends of oligonucleotide to allow the amplified fragment to be digested with XhoI, and then to be ligated into the XhoI site of pET-UBC4. This insertion created an in frame fusion of UBC4 protein with the ProteinA D domain and the resulting plasmid was designated pET-UBC4-ProteinA. The presence of antibody-binding domain in the expressed pET-UBC4-ProteinA construct was demonstrated by expression in *E. coli* and immunoblot analysis using alkaline phosphatase-conjugated immunoglobulin G. The resulting fusion protein is schematically illustrated in Fig. 6.

### 2. Ubiquitin conjugation assays.

#### A. Expression and assay of the UBC1 and UBC4 constructs.

All pET3a expression plasmids containing the UBC1 and UBC4 expression cassettes were transformed into *E. coli* strain BL21(DE3). Following induction of pET3a expression cassette by the addition of isopropyl



$\beta$ -D-thiogalactopyranoside to logarithmic growth phase cultures, the cells were harvested and lysed as described in Sullivan and Vierstra, (1991) *supra*. All experiments were performed using crude lysates of cells containing the induced plasmids. Ubiquitin conjugation assays with these lysates were performed as previously described in Sullivan and Vierstra, (1991) *supra*, except that the incubation time for all assays was 2 hours. Each of the reactions was formed in 20  $\mu$ l total volume containing 1-4  $\mu$ l of bacterial extracts harboring the expressed E2 fusion protein molecules, 12  $\mu$ g/ml of purified E1, 0.52  $\mu$ g of  $^{125}$ I-ubiquitin, 1 unit of inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) in 20  $\mu$ l of 50 mM Tris (pH 7.6 at 25°C), 10 mM MgCl<sub>2</sub>, 1 mM ATP, 0.1 mM dithiothreitol and varying concentrations of the substrate. Prior to performing these conjugation assays, the activity of each E2 fusion molecule was determined by its ability to accept activated ubiquitin from E1 alone and bind to the ubiquitin via a thiol-ester bond by the method described in Sullivan and Vierstra, (1991) *supra*. Based on this thiol-ester assay, the volume of bacterial extracts that contained equivalent amounts of E2 activity was determined for each construction. This normalized volume was added to the various conjugation assays. The conjugation assays were terminated by adding an equal volume of 25 mM Tris-HCl (pH 6.8), 5% (v/v) glycerol, 4% (w/v) sodium dodecyl sulfate, 10% (v/v) 2-mercaptoethanol to the reactions and heating the mixture to 100°C for 5 minutes. Samples were subjected to SDS-PAGE using the system of Laemmli, *Nature*, 227:680 (1970). The gels were then stained with Coomassie Blue, dried between sheets of cellophane, and used for autoradiography. This is intended to visualize the size of any proteins which have bound the radiolabeled ubiquitin to thus indicate if the radiolabeled ubiquitin molecule has been properly attached to the target protein of the expected size.

#### B. Conjugation of ubiquitin to immunoglobulins in the presence of eukaryotic extracts.

The formation of ubiquitin-antibody conjugates in the presence of wheat germ extracts or rabbit reticulocyte lysates were as described by Hatfield and Vierstra (1989), *supra*, for wheat germ. The reaction mixtures for these reactions were 20  $\mu$ l total, containing 12  $\mu$ g/ml of purified wheat E1, 50  $\mu$ g/ml of human ubiquitin, 100  $\mu$ g/ml of anti-S3 mono-clonal antibody, 30 units/ml of creatine kinase, and 4  $\mu$ l of wheat germ extract or rabbit reticulocyte lysate (Promega) in 80 mM Tris (pH 8.5 at 25°C), 20 mM creatine phosphate, 7.5 mM MgCl<sub>2</sub>, 2 mM ATP, and 2 mM dithiothreitol. Each of the reactions was initiated by adding 2  $\mu$ l of E2 fusion protein extracts and incubated at 30°C for the indicated time periods. The reactions were terminated in the same manner as the expression conjugation assays described above. The samples were subjected to SDS-PAGE and the proteins were electroblotted to Immobilon-P. The antibody-conjugates were identified by immunoblot analysis using alkaline phosphatase-conjugated goat-anti-mouse immunoglobulins as described previously in Sullivan and Vierstra, *supra*.

#### C. Results of Conjugation Assays.

It was determined that in all cases *E. coli* was capable of expressing the chimeric UBC1 and UBC4 genes and to synthesize the synthetic E2 fusion proteins of the expected and desired sizes. All of E2 fusion proteins thus created were enzymatically active, based on their ability to interact with ubiquitin via the formation of a thiol ester bond to ubiquitin. When unmodified UBC1 or UBC4 was tested in ubiquitin conjugation assays, little or no conjugation was observed to the desired substrate tested. Conversely, when the appropriate fusions were tested, highly specific conjugation to the various substrates was observed, as demonstrated by appropriately sized bands on the radiolabeled blots indicating that proteins of the expected size were tagged with the radiolabeled ubiquitin. Thus the conjugation was detected by the attachment of free  $^{125}$ I-labeled ubiquitin to the target via a peptide bond. The formation of such ubiquitin-protein conjugates was visualized by the autoradiography as a mobility shift during the SDS-page analysis of ubiquitin from that of the free form to that of a protein expected to contain both ubiquitin and a substrate protein.

As an example, whereas UBC4 could not conjugate ubiquitin to the c-myc monoclonal antibody, the fusion protein expressed by the plasmid pET-UBC4-(c-myc) (Fig. 3) could in fact conjugate ubiquitin to the c-myc monoclonal antibody. In this particular experiment, a single ubiquitin was added to the heavy chain of the antibody. The ubiquitin-antibody conjugate migrated at the expected molecular mass of the heavy chain of the antibody (55 kDa) with the addition of a single ubiquitin moiety (6 kDa). The presence of the antibody in the conjugate was confirmed by its immunorecognition by ProteinA. The reaction was judged to be specific for the c-myc antibody based on the fact that other mouse monoclonal antibodies failed to be conjugated by the E2 fusion protein expressed by the pET-UBC4-(c-myc) vector and also by the fact that addition of excess c-myc peptide blocked the antibody conjugation reaction.

Using the UBC1 E2 variant in which the c-myc epitope was fused directly to the UBC1 core region, attempts to perform similar experiments to add an ubiquitin conjugate to an antibody failed. Other experiments had hint-

ed that perhaps a spacer arm, already present in UBC4, was required. Accordingly, the construct described above designated pET-UBC1-spacer-(c-myc) was constructed to test whether an artificial spacer arm would suffice to render the UBC1 conjugate active. When the fusion protein expressed by the pET-UBC1-spacer-(c-myc) expression cassette was tested, it was found capable of specifically conjugating the radiolabeled ubiquitin to the c-myc monoclonal antibody. This result demonstrated that the protein binding ligand on the fusion protein which is specific to the target protein must be physically placed beyond the body of the E2 core region in such a fusion protein. As with UBC4-(c-myc), ubiquitin conjugated by the fusion protein including UBC1-spacer-(c-myc) was specific for the c-myc antibody, and it did not modify other mouse monoclonal antibodies. The fusion protein was also blocked by the addition of excess free-c-myc peptide to the reaction mixture.

To further test the ability of the system to conjugate ubiquitin to monoclonal antibodies, the construct including the domain D from ProteinA was also tested. The E2 fusion protein created by the plasmid designated pET-UBC4-ProteinA was added to conjugation reactions containing purified antibodies that naturally interact with ProteinA. In these reactions, attachment of the ubiquitin moiety to the heavy chain of the antibodies was observed. In contrast, no such conjugation was observed when wild-type UBC4 was used in similar reactions. The ability to attached ubiquitin to the antibody correlated with the known affinity of ProteinA to the various antibody classes. For example, whereas mouse immunoglobulin G, which binds to ProteinA tightly, were quite effectively conjugated by the E2 fusion protein containing UBC4 and ProteinA, an immunoglobulin G from goat, which normally binds weakly if at all to ProteinA, was not similarly conjugated. Again the results were determined by radiolabeled blotting.

The interaction of the GeneV protein, which associates with itself into homodimers, which homodimers in turn bind cooperatively with single-stranded DNA, was also tested utilizing the UBC4-GeneV construct. First it was determined that a construct expressing the UBC4 protein itself was unable to conjugate ubiquitin to GeneV protein. Similar experiments were performed using the E2 fusion proteins created by the plasmid pET-UBC4-GeneV. That fusion protein was found capable of creating ubiquitin-GeneV conjugates. In this case, the recognition of GeneV is apparently accomplished by the dimerization between the wild-type GeneV and the GeneV domain of the E2 fusion protein consisting of both UBC4 and GeneV. This demonstrates that dimerization-type affinities between the protein binding domain of the E2 fusion molecules constructed in accordance with the present invention will sufficiently bind to target proteins so as to allow them to be the target for ubiquitin fusion.

To test the ability of the E2 fusion protein strategy described herein to conjugate ubiquitin to hormones or receptors, the E2 fusion protein consisting of UBC4 and TGF $\alpha$  was utilized. Both TGF $\alpha$  and the related peptide hormone, epidermal growth factor, are capable of highly specific and very tight binding to the EGF receptor. When the E2 fusion protein expressed by the plasmid pET-UBC4-TGF $\alpha$  was added to crude human epidermal membranes, the result was highly specific modification of the EGFR protein by conjugation with ubiquitin. This conjugation was specific for the TGF $\alpha$ -EGFR pair as judged by the failure of unmodified UBC4 protein to ubiquitinate the receptor and the ability of free, excess, epidermal growth factor in the reaction mixture to block the reaction and prevent ubiquitination of the EGF receptor. The result also clearly demonstrates that species differentiations between E2s and ubiquitins are not critical to this reaction since the UBC4 plant origin is quite clearly capable of ubiquitinating the target molecule of mammalian origin in this reaction. Since M13 is a bacteriophage, the same phenomenon can be used on bacterial targets as well.

It has been observed that selective proteolytic degradation by the ubiquitin-directed protein degradation pathway appears to involve the conjugation of multiple ubiquitins to the target protein, in many cases forming a multiubiquitin chain. In the conjugation assays described above, using the bacterial-expressed E2s, generally only attachment of a single ubiquitin to the target molecule was detected in most cases. As a result, it was possible to assert that ubiquitination by the E2 carboxyl-terminal fusion proteins described herein would not form the multiubiquitinated intermediates necessary to cause the targeted protein to enter into the degradative pathway.

To test whether that limitation was a real one, experiments were conducted in which crude eukaryotic cell extracts, either rabbit reticulocyte extract or wheat germ extract, were added to the ubiquitin conjugation assays. Since such crude extracts often contain endogenous multiubiquitin chains, the idea was to test to see if such multiubiquitin chains could be added by the E2 fusion proteins described herein to the target molecule. Such attachment of multiubiquitin chains to the target molecules was observed. For example, in the absence of such extracts, only a single ubiquitin becomes attached to mouse immunoglobulin G in the presence of E2 fusion protein consisting of UBC4-ProteinA. But upon the addition of either wheat germ extract or rabbit reticulocyte extracts to the reaction mixture, the same system was capable of generating ubiquitinated forms of mouse immunoglobulin G with as many as 7-8 ubiquitin repeats attached to the antibody. Based on available evidence to date, such heavily modified forms represent acceptable substrates for subsequent degradation by the ubiquitin system and are highly likely to be recognized by that system and then subjected to proteolytic

degradation. This results demonstrate that the E2 fusion proteins described herein are capable of generating proteolytic intermediates with the help of other endogenous factors normally present within eukaryotic cells.

#### Hypothetical Example

The experiments above demonstrate that the specificity of the ubiquitin conjugation can be modified in predetermined ways to target new proteins for degradation. The versatility of this approach depends on identifying a protein binding domain which can be attached to the E2 fusion molecule which will then bind to the protein of interest. Obviously, because the information is limited as to the nature of the interaction between many proteins and other proteins within the cell, the use of natural protein-protein interactions would restrict the technology present to only a few well characterized types. However, the exploitation of antibody/antigen reactions has the power to overcome this obstacle. It is possible, of course, to create antibodies which bind for most specific proteins of interest. The binding domains (Fab) of such antibodies, which involve amino acids from both the heavy and light immunoglobulin chains, can now be identified and expressed as single shorter peptides which are referred to as single chain monoclonal antibodies. The genes for the single chain monoclonal antibodies express Fab region fragments linked by a shorter flexible spacer region. This concept is illustrated in Fig. 7. It is intended that such single chain monoclonal antibodies can be fused to the carboxyl terminus of E2s like UBC4, to create E2 fusion proteins which can be targeted through the Fab region to any protein of interest for which a monoclonal antibody is either available or can be developed. There has recently been commercialized a kit for constructing this type of antibody which allows the facile development of single chain antibodies genes against any suitable antigenic protein. The availability of this technology suggests that E2 fusion proteins with an Fab protein ligand binding region can be constructed to target virtually any proteins for degradation using this system.

## SEQUENCE LISTING

## 5 (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: WISCONSIN ALUMNI RESEARCH FOUNDATION

(B) STREET: P.O. Box 7365

10 (C) CITY: Madison

(D) STATE: Wisconsin

(E) COUNTRY: U.S.A.

(F) POSTAL CODE (ZIP): 53707-7365

15 (ii) TITLE OF INVENTION: UBIQUITIN CONJUGATING ENZYME (E2) FUSION  
PROTEINS

(iii) NUMBER OF SEQUENCES: 18

## (iv) COMPUTER READABLE FORM:

20 (A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

25 (v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: EP 94303903.2

## 30 (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 757 base pairs

(B) TYPE: nucleic acid

35 (C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

40 (A) NAME/KEY: CDS

(B) LOCATION: 100..558

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

45 CGGCGGTCAA CACCGCTGAA CACATATGAA AGAAAGACGA CCTCTTCTCT CCGCGATCTT 60  
TACCTCAACA ACGAGATCTG TTTCCAGAAA GAAAGGAGG ATG TCG ACG CCA GCA 114  
Met Ser Thr Pro Ala  
1 5

50 AGG AAG AGG TTA ATG AGG GAT TTC AAG AGG TTG CAG CAA GAC CCA CCT 162  
Arg Lys Arg Leu Met Arg Asp Phe Lys Arg Leu Gln Gln Asp Pro Pro  
10 15 20

55 GCG GGT ATT AGT GGT GCT CCA CAG GAC AAC AAC ATT ATG CTC TGG AAT 210  
Ala Gly Ile Ser Gly Ala Pro Gln Asp Asn Asn Ile Met Leu Trp Asn  
25 30 35

GCT GTC ATA TTT GGG CCT GAT GAC ACA CCA TGG GAT GGA GGT ACT TTC 258  
 Ala Val Ile Phe Gly Pro Asp Asp Thr Pro Trp Asp Gly Gly Thr Phe  
 40 45 50  
 5 AAA CTC TCA CTG CAG TTC TCT GAA GAT TAT CCC AAT AAA CCA CCA ACA 306  
 Lys Leu Ser Leu Gln Phe Ser Glu Asp Tyr Pro Asn Lys Pro Pro Thr  
 55 60 65  
 10 GTT CGG TTT GTG TCA CGG ATG TTT CAT CCT AAT ATT TAT GCA GAT GGG 354  
 Val Arg Phe Val Ser Arg Met Phe His Pro Asn Ile Tyr Ala Asp Gly  
 70 75 80 85  
 15 AGT ATC TGC TTG GAC ATT CTA CAA AAC CAG TGG AGT CCA ATC TAT GAT 402  
 Ser Ile Cys Leu Asp Ile Leu Gln Asn Gln Trp Ser Pro Ile Tyr Asp  
 90 95 100  
 GTT GCT GCT ATA CTT ACC TCC ATC CAG TCC TTG CTC TGT GAC CCT AAT 450  
 Val Ala Ala Ile Leu Thr Ser Ile Gln Ser Leu Leu Cys Asp Pro Asn  
 105 110 115  
 20 CCG AAT TCT CCT GCA AAC TCG GAA GCT GCT CGG ATG TAC AGC GAA AGC 498  
 Pro Asn Ser Pro Ala Asn Ser Glu Ala Ala Arg Met Tyr Ser Glu Ser  
 120 125 130  
 25 AAG CGC GAG TAC AAC AGG AGA GTG CGT GAT GTT GTT GAG CAA AGC TGG 546  
 Lys Arg Glu Tyr Asn Arg Arg Val Arg Asp Val Val Glu Gln Ser Trp  
 135 140 145  
 30 ACT GCT GAC TAGTAGTAGT TTGTTGTAAG CGTTGTAGCT CTCTCTACTT 595  
 Thr Ala Asp  
 150  
 TCTCTCAATC ACGATTGAGC AACAGCTTTC TTCTCTTTTC ATTCATGTCT TGTGTTTCCA 655  
 35 AAATATTTTA AGTGATTCCA TGCTTTGATG TAACCCAACA TCCTTAAAAA AACAACTTTG 715  
 TACCAAACCA TCTGAATTAT TCACTTTTGT GTATAAAAAA AA 757

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 152 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

50 Met Ser Thr Pro Ala Arg Lys Arg Leu Met Arg Asp Phe Lys Arg Leu  
 1 5 10 15  
 Gln Gln Asp Pro Pro Ala Gly Ile Ser Gly Ala Pro Gln Asp Asn Asn  
 20 25 30  
 55 Ile Met Leu Trp Asn Ala Val Ile Phe Gly Pro Asp Asp Thr Pro Trp  
 35 40 45

Asp Gly Gly Thr Phe Lys Leu Ser Leu Gln Phe Ser Glu Asp Tyr Pro  
 50 55 60  
 5 Asn Lys Pro Pro Thr Val Arg Phe Val Ser Arg Met Phe His Pro Asn  
 65 70 75 80  
 Ile Tyr Ala Asp Gly Ser Ile Cys Leu Asp Ile Leu Gln Asn Gln Trp  
 85 90 95  
 10 Ser Pro Ile Tyr Asp Val Ala Ala Ile Leu Thr Ser Ile Gln Ser Leu  
 100 105 110  
 Leu Cys Asp Pro Asn Pro Asn Ser Pro Ala Asn Ser Glu Ala Ala Arg  
 115 120 125  
 15 Met Tyr Ser Glu Ser Lys Arg Glu Tyr Asn Arg Arg Val Arg Asp Val  
 130 135 140  
 Val Glu Gln Ser Trp Thr Ala Asp  
 145 150  
 20

## (2) INFORMATION FOR SEQ ID NO:3:

25 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 980 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

(ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 60..614  
 35

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGAATTCCCA AACCTACAAG CAGGGCAAGG AGGAGGAGGA AGAAGAAGAA GAAGCAAAC 59  
 40 ATG TCT TCC CCA AGC AAG CGC AGG GAG ATG GAT CTC ATG AAG CTG ATG 107  
 Met Ser Ser Pro Ser Lys Arg Arg Glu Met Asp Leu Met Lys Leu Met  
 1 5 10 15  
 ATG AGT GAC TAC AAG GTG GAC ATG ATC AAC GAC GGG ATG CAC GAG TTC 155  
 45 Met Ser Asp Tyr Lys Val Asp Met Ile Asn Asp Gly Met His Glu Phe  
 20 25 30  
 TTC GTC CAC TTC CAC GGA CCC AAA GAC AGT ATT TAC CAG GGT GGT GTG 203  
 50 Phe Val His Phe His Gly Pro Lys Asp Ser Ile Tyr Gln Gly Gly Val  
 35 40 45  
 TGG AAG GTC AGG GTT GAA CTC ACC GAA GCT TAC CCT TAC AAA TCC CCT 251  
 Trp Lys Val Arg Val Glu Leu Thr Glu Ala Tyr Pro Tyr Lys Ser Pro  
 50 55 60  
 55 TCC ATT GGC TTC ACC AAC AAG ATC TAT CAC CCC AAT GTC GAT GAG ATG 299  
 Ser Ile Gly Phe Thr Asn Lys Ile Tyr His Pro Asn Val Asp Glu Met

	65				70				75				80									
5	TCT Ser	GGT Gly	TCT Ser	GTC Val	TGC Cys 85	TTG Leu	GAT Asp	GTG Val	ATC Ile	AAT Asn 90	CAG Gln	ACA Thr	TGG Trp	AGC Ser	CCG Pro 95	ATG Met	347					
10	TTT Phe	GAC Asp	CTT Leu	GTG Val 100	AAT Asn	ATC Ile	TTT Phe	GAG Glu	GTG Val 105	TTC Phe	CTG Leu	CCC Pro	CAG Gln	CTT Leu 110	CTC Leu	CTG Leu	395					
15	TAC Tyr	CCG Pro	AAC Asn 115	CCC Pro	TCG Ser	GAC Asp	CCC Pro	TTG Leu 120	AAC Asn	GGC Gly	GAG Glu	GCG Ala	GCT Ala 125	TCG Ser	CTC Leu	ATG Met	443					
20	ATG Met 130	CGC Arg	GAC Asp	AAG Lys	AAT Asn	GCC Ala	TAT Tyr 135	GAA Glu	AAT Asn	AAA Lys	GTC Val 140	AAA Lys	GAA Glu	TAT Tyr	TGT Cys	GAG Glu	491					
25	AGA Arg 145	TAT Tyr	GCC Ala	AAG Lys	CCT Pro	GAA Glu 150	GAT Asp	ATA Ile	TCC Ser	CCA Pro	GAG Glu 155	GAG Glu	GAA Glu	GAG Glu	GAG Glu	GAG Glu 160	539					
30	AGT Ser	GAT Asp	GAG Glu	GAG Glu	CTG Leu 165	AGC Ser	GAC Asp	GCC Ala	GAG Glu	GGC Gly 170	TAC Tyr	GAC Asp	TCC Ser	GGC Gly 175	GAC Asp	GAG Glu	587					
35	GCC Ala	ATC Ile	ATG Met	GGC Gly	CAC His 180	GCA Ala	GAC Asp	CCT Pro	TAAGTGGTGG 185				ATGGATGCAA GGATGGTTAG				641					
40	CTCAGTCAGT				AACTCAGTAA				TGCAGGTGAT				CATGATGTAT				CTCTGTCTGT				CAGTCTGTAC	701
	ATAGCTGCGG				CGATCACTGA				TGAATGCCGC				CATGGCAGAT				GCTGAAGAAA				GTCATCAGCC	761
	ATCTCAACTC				AGCTCCACTA				GTTCTTGTGT				GTCCCGCTGT				GAATAACTTG				CCATTTGTTT	821
	GTGTTGGTTC				CATTTGCAGT				TCATGTTTCC				ATTCTAGGAG				ATGTCTGTTC				TTCTGTTTTG	881
	TTGATTTTCAT				TTCCAGTTCA				TGTTACTACT				GTATGTTTCC				CTTTCCTACC				TGTAATCATC	941
	TCAGGGGAAT				TTAAATCTGC				TCTGCATGTC				CAGGAATTC									980

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 184 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ser Ser Pro Ser Lys Arg Arg Glu Met Asp Leu Met Lys Leu Met  
 1 5 10 15  
 Met Ser Asp Tyr Lys Val Asp Met Ile Asn Asp Gly Met His Glu Phe

**EP 0 626 450 A2**

[illegible]

(2) INFORMATION FOR SEQ ID NO:5:

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 37 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

45 GGCCACGCAG ACCCTCTCGA GTAGGATGGA TGCAAGG

(2) INFORMATION FOR SEQ ID NO:6:

50 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 41 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:



GCAAAGCTGG ACTGCTCTCG AGTAGTAGTT TGTTGTAAGC G

41

## (2) INFORMATION FOR SEQ ID NO:7:

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- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 39 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

10

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 6..35

15

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCGAG GAG CAG AAG CTG ATC AGC GAG GAG GAC CTG TAAC  
 Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu  
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20

39

## (2) INFORMATION FOR SEQ ID NO:8:

25

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 10 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

30

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu  
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35

## (2) INFORMATION FOR SEQ ID NO:9:

40

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 39 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

45

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCGAGTTACA GGTCCCTCCTC GCTGATCAGC TTCTGCTCC

39

50

## (2) INFORMATION FOR SEQ ID NO:10:

55

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 45 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:  
 CCCGCCCGTG GCTGCACTCG AGGTGTCCCA TTTAATGAC TGCCC 45  
 (2) INFORMATION FOR SEQ ID NO:11:  
 10 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 49 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 15  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:  
 20 GGCCTGCTTC TTCTGGCTGG CGTCGACCTA GGCCAGGAGG TCCGCATGC 49  
 (2) INFORMATION FOR SEQ ID NO:12:  
 25 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 35 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 30  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:  
 AGGTAACTCG AGATGATTAA AGTTGAAATT AAACC 35  
 35 (2) INFORMATION FOR SEQ ID NO:13:  
 40 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 34 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 45  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:  
 CGACCTGGTC GACGTTACTT AGCCGGAACG AGGC 34  
 (2) INFORMATION FOR SEQ ID NO:14:  
 50 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 37 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTTAATGACC TCGAGGCTCC AAAAGCTGAT GCGCAAC

37

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 37 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTTGAAATTC TCGAGTTATT TCGGTGCTTG AGATTCTG

37

## (2) INFORMATION FOR SEQ ID NO:16:

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 39 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10 (ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 3..32

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TC GAA CCA CCA GTC GAC GCA GCA GCA GCA GCA CTCGAGT 39  
Glu Pro Pro Val Asp Ala Ala Ala Ala Ala  
1 5 10

20

## (2) INFORMATION FOR SEQ ID NO:17:

25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Glu Pro Pro Val Asp Ala Ala Ala Ala Ala  
1 5 10

35

## (2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 39 base pairs  
(B) TYPE: nucleic acid  
40 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCGAACTCGA GTGCTGCTGC TGCTGCGTCG ACTGGTGGT 39

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## Claims

- 5 1. A DNA sequence encoding a ubiquitin-conjugating E2 fusion protein, the DNA sequence comprising:  
a promoter effective in the cells of a host to express a protein coding sequence located 3' to the promoter; and  
a protein coding sequence located 3' to the promoter, the protein coding sequence including 5' to  
3':  
10 a DNA coding sequence encoding a ubiquitin-conjugating E2 core region;  
a DNA coding sequence encoding a spacer of at least four amino acids; and  
a DNA coding sequence encoding a heterologous protein binding ligand having affinity for a target  
protein.
- 15 2. A DNA sequence according to claim 1 wherein the E2 core region is from a plant E2 gene.
3. A DNA sequence according to claim 2 wherein the E2 core region is selected from the group consisting  
of the core regions of UBC1, shown in SEQ:ID:NO:1 and UBC4, shown in SEQ:ID:NO:3.
- 20 4. A DNA sequence according to any one of the preceding claims wherein the DNA coding sequences for  
both the core region and the spacer are from a single native E2 coding region.
5. A DNA sequence according to claim 4 wherein the DNA coding sequences for both the core region and  
the spacer are from UBC4, shown in SEQ:ID:NO:3.
- 25 6. A DNA sequence according to any one of claims 1 to 3 wherein the spacer is an artificial amino acid se-  
quence.
7. A DNA sequence according to any one of the preceding claims wherein the protein binding ligand is a rec-  
ognition domain of an antibody, the domain having binding specificity for the target protein.
- 30 8. A DNA sequence as claimed in any one of claim 1 to 6 wherein the protein binding ligand is a domain of  
protein A having binding specificity for antibodies.
9. A DNA sequence according to any one of claims 1 to 6 wherein the protein binding ligand is selected from  
the group consisting of protein hormones and cellular receptors for protein hormones.
- 35 10. A DNA sequence according to any one of claims 1 to 6 wherein the protein binding ligand is an epitope  
recognized by an antibody.
- 40 11. A replication and expression vector comprising a DNA sequence according to any one of the preceding  
claims.
12. Host cells transformed or transfected with a replication and expression vector according to claim 11.
13. A non-human transgenic eukaryotic organism comprising in its genome the DNA of any one of claim 1 to  
45 10.
14. A non-human transgenic eukaryotic organism as claimed in claim 13 wherein the organism is a plant.
- 50 15. A method of producing a ubiquitin-conjugating E2 fusion protein which comprises culturing host cells ac-  
cording to claim 12 under conditions effective to express a ubiquitin-conjugate E2 fusion protein compris-  
ing, from amino to carboxyl terminus:  
a core region derived from a ubiquitin-conjugating E2 protein and which is functionally able to form  
a thiol linkage to a ubiquitin protein by transesterification from an E1 protein;  
a spacer of at least four amino acids; and  
55 a heterologous protein binding ligand having specific binding affinity for at least one target protein,  
so that the entire E2 fusion protein will be capable of forming a thiol linkage to at least one ubiquitin moiety,  
binding to target protein, and transferring the ubiquitin moiety into a covalent bond to the target protein.

16. A ubiquitin-conjugating E2 fusion protein comprising, from amino to carboxyl terminus:  
 a core region derived from a ubiquitin-conjugating E2 protein and which is functionally able to form  
 a thiol linkage to a ubiquitin protein by transesterification from an E1 protein;  
 a spacer of at least four amino acids; and  
 a heterologous protein binding ligand having specific binding affinity for at least one target protein,  
 so that the entire E2 fusion protein will be capable of forming a thiol linkage to at least one ubiquitin moiety,  
 binding to target protein, and transferring the ubiquitin moiety into a covalent bond to the target protein.
17. A protein according to claim 16 which is encoded by a DNA as claimed in any one of claims 2 to 10.
18. A non-human transgenic eukaryotic organism comprising the E2 fusion protein of claim 16.
19. A non-human transgenic eukaryotic organism as claimed in claim 18 wherein the organism is a plant.
20. A method of causing the addition of a ubiquitin moiety to a target protein comprising the steps of  
 (a) constructing a DNA sequence for a ubiquitin-conjugating E2 fusion protein including:  
 a promoter effective in the cells of a host to express a protein coding sequence located 3' to the  
 promoter; and  
 a protein coding sequence located 3' to the promoter, the protein coding sequence including 5' to  
 3':  
 a DNA coding sequence encoding a ubiquitin-conjugating E2 core region;  
 a DNA coding sequence encoding a spacer of at least four amino acids; and  
 a DNA coding sequence encoding a heterologous protein binding ligand having affinity for the  
 target protein;  
 (b) transforming the DNA sequence from step (a) into a host in which the promoter is capable of causing  
 expression of the E2 fusion protein, so that the E2 fusion protein is produced in the host; and  
 (c) exposing the E2 fusion protein to ubiquitin-linked E1, and a source of energy so that the E2 fusion  
 protein will accept the ubiquitin from the E1 and transfer the ubiquitin specifically to the target protein  
 if present.
21. A method according to claim 20 wherein transformation is performed in a host containing the target protein  
 and the fusion protein is exposed to the ubiquitin-linked E1 by in vivo expression of the E2 fusion protein  
 in the cells of the host.
22. A method according to claim 20 wherein the target protein is not in the host in which the fusion protein is  
 expressed and the method further comprises recovering the E2 fusion protein expressed in the host and  
 introducing the E2 fusion protein into a second host in which the target protein is present.
23. A method according to claim 20, 21 or 22 in which the degradation of a target protein is directed by ex-  
 posing the E2 fusion protein to ubiquitin-linked E1, a source of energy, and a proteosome proteolytic com-  
 plex, so that the E2 fusion protein will accept the ubiquitin from the E1 and transfer the ubiquitin specif-  
 ically to the target protein directing degradation of the target protein by the proteosome.
24. A method of directing the degradation of a target protein in a host organism comprising the steps of  
 (a) constructing a DNA sequence for a ubiquitin-conjugating E2 fusion protein including:  
 a promoter effective in the cells of a host to express a protein coding sequence located 3' to the  
 promoter; and  
 a protein coding sequence located 3' to the promoter, the protein coding sequence including,  
 5' to 3':  
 a DNA coding sequence encoding a ubiquitin-conjugating E2 core region;  
 a DNA coding sequence encoding a spacer of at least four amino acids; and  
 a DNA coding sequence encoding a heterologous protein binding ligand having affinity for the  
 target protein; and  
 (b) transforming the DNA sequence from step (a) into the host organism so that the E2 fusion protein  
 will add a ubiquitin moiety to the target protein to direct the degradation of the target protein by a cellular  
 protein degrading pathway.
25. A method according to claim 24 wherein the host is a plant.

26. A method of causing the degradation of a target protein in a target host comprising the steps of
- (a) constructing a DNA sequence for a ubiquitin-conjugating E2 fusion protein including:
    - a promoter effective in the cells of a host to express a protein coding sequence located 3' to the promoter; and
    - 5 a protein coding sequence located 3' to the promoter, the protein coding sequence including, 5' to 3':
      - a DNA coding sequence encoding a ubiquitin-conjugating E2 core region;
      - a DNA coding sequence encoding a spacer of at least four amino acids; and
      - a DNA coding sequence encoding a heterologous protein binding ligand having affinity for the
  - 10 target protein;
  - (b) transforming the DNA sequence from step (a) into a production host in which the promoter is capable of causing expression of the E2 fusion protein, so that the E2 fusion protein, so that the E2 fusion protein is produced in the production host;
  - (c) recovering the expressed E2 fusion protein from the production host; and
  - 15 (d) introducing the recovered E2 fusion protein into the target host.
27. A method according to claim 26 wherein the target host is a mammal.

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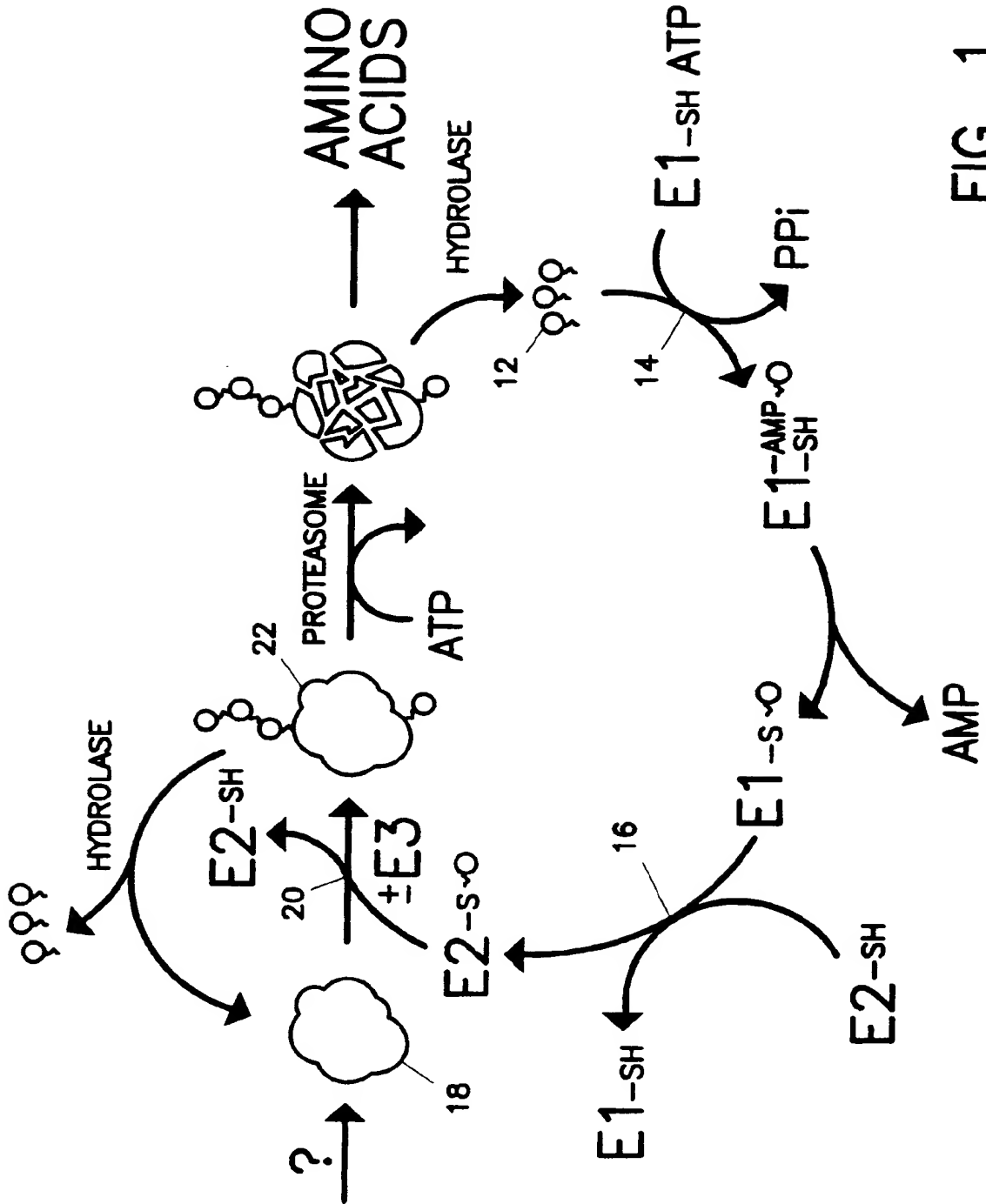


FIG. 1



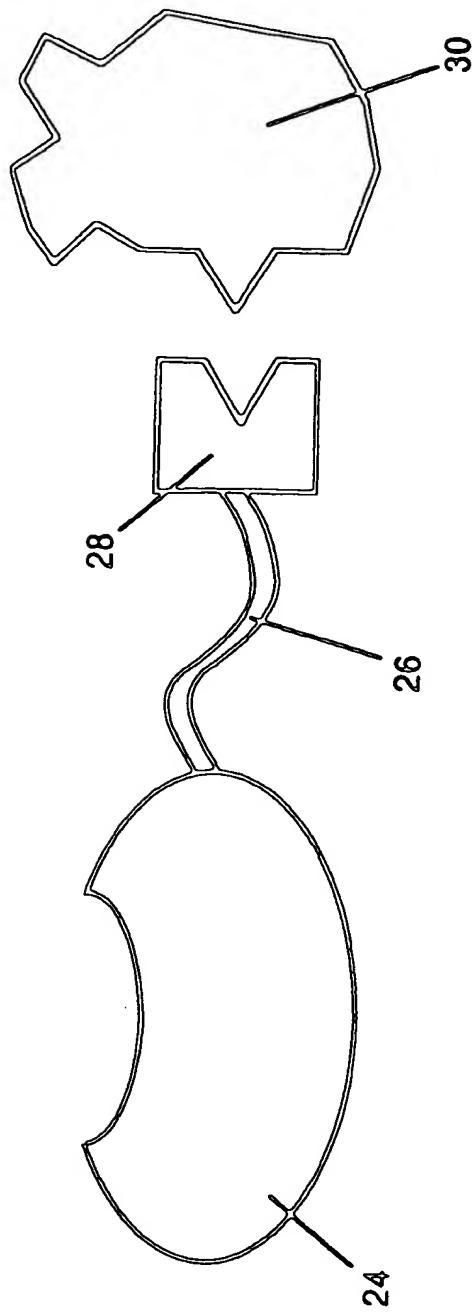


FIG. 2

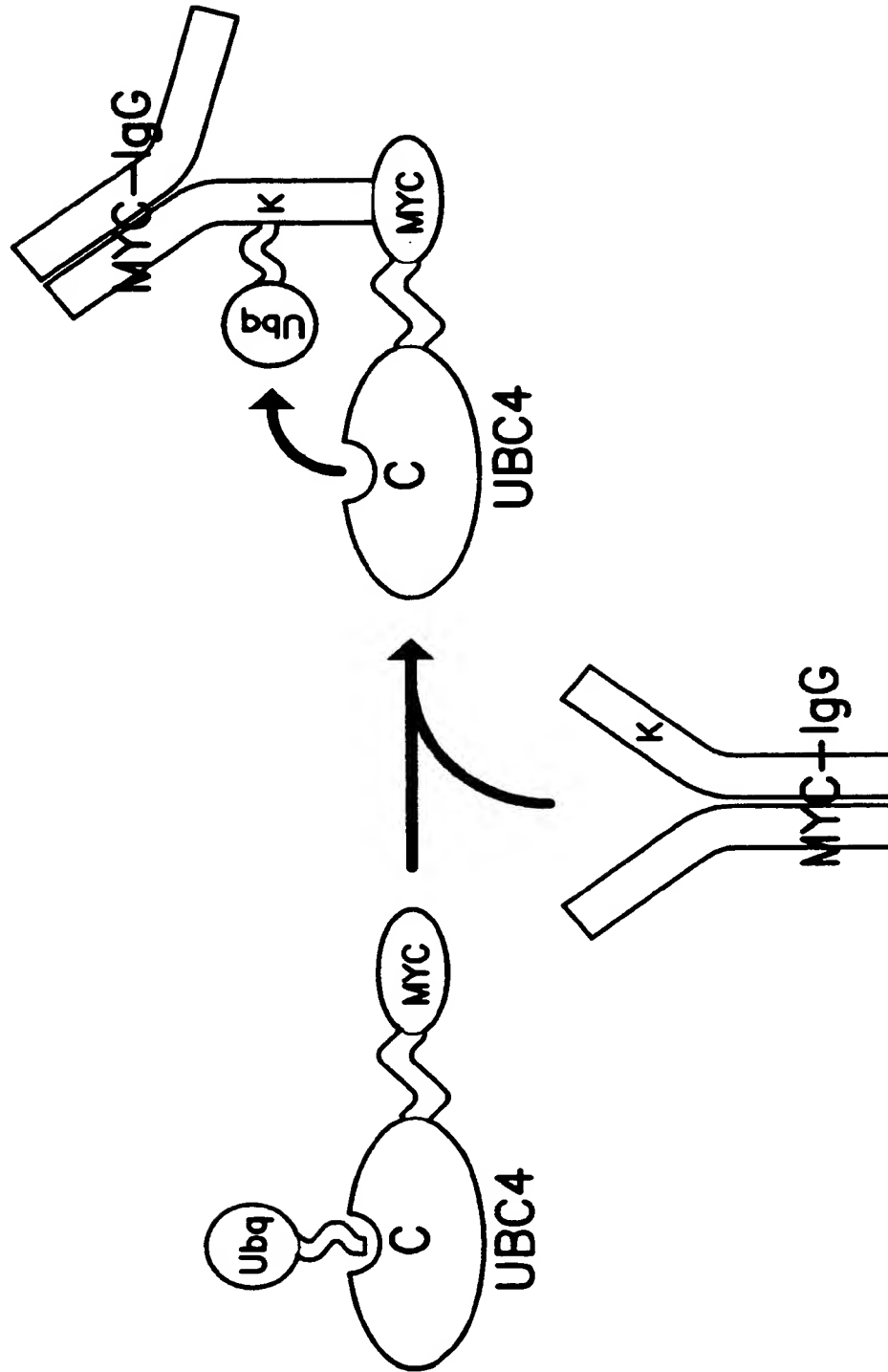


FIG. 3

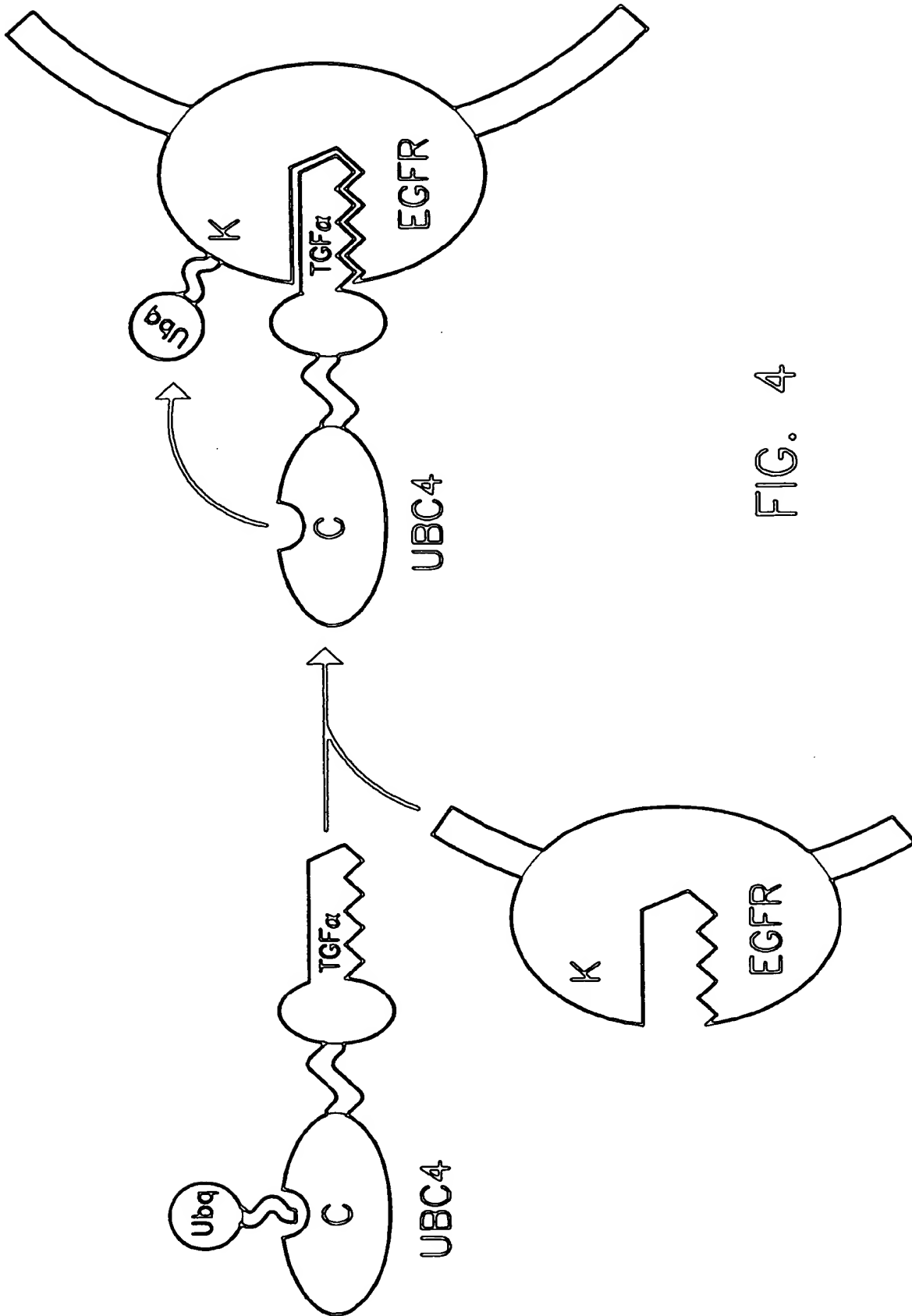


FIG. 4

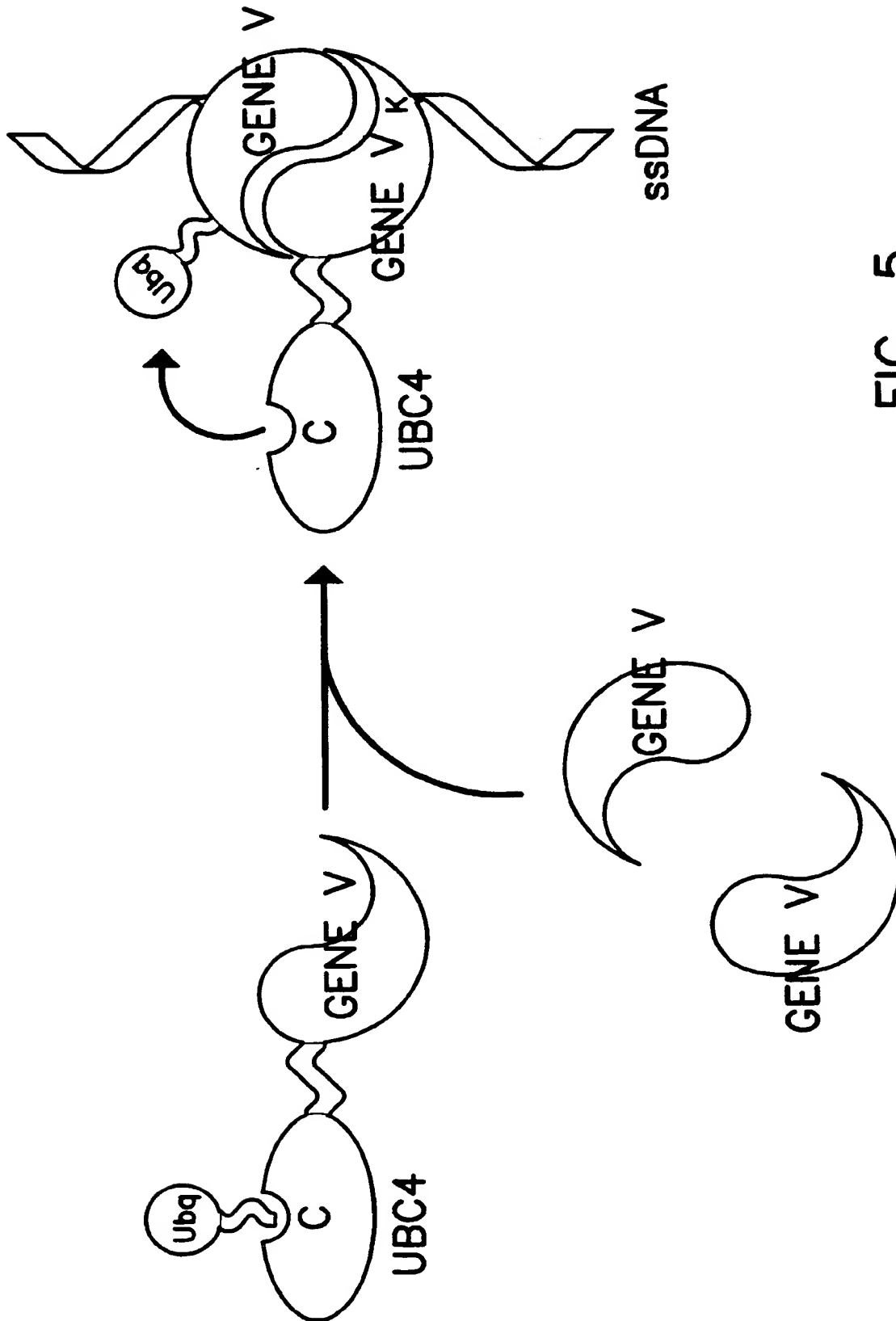


FIG. 5

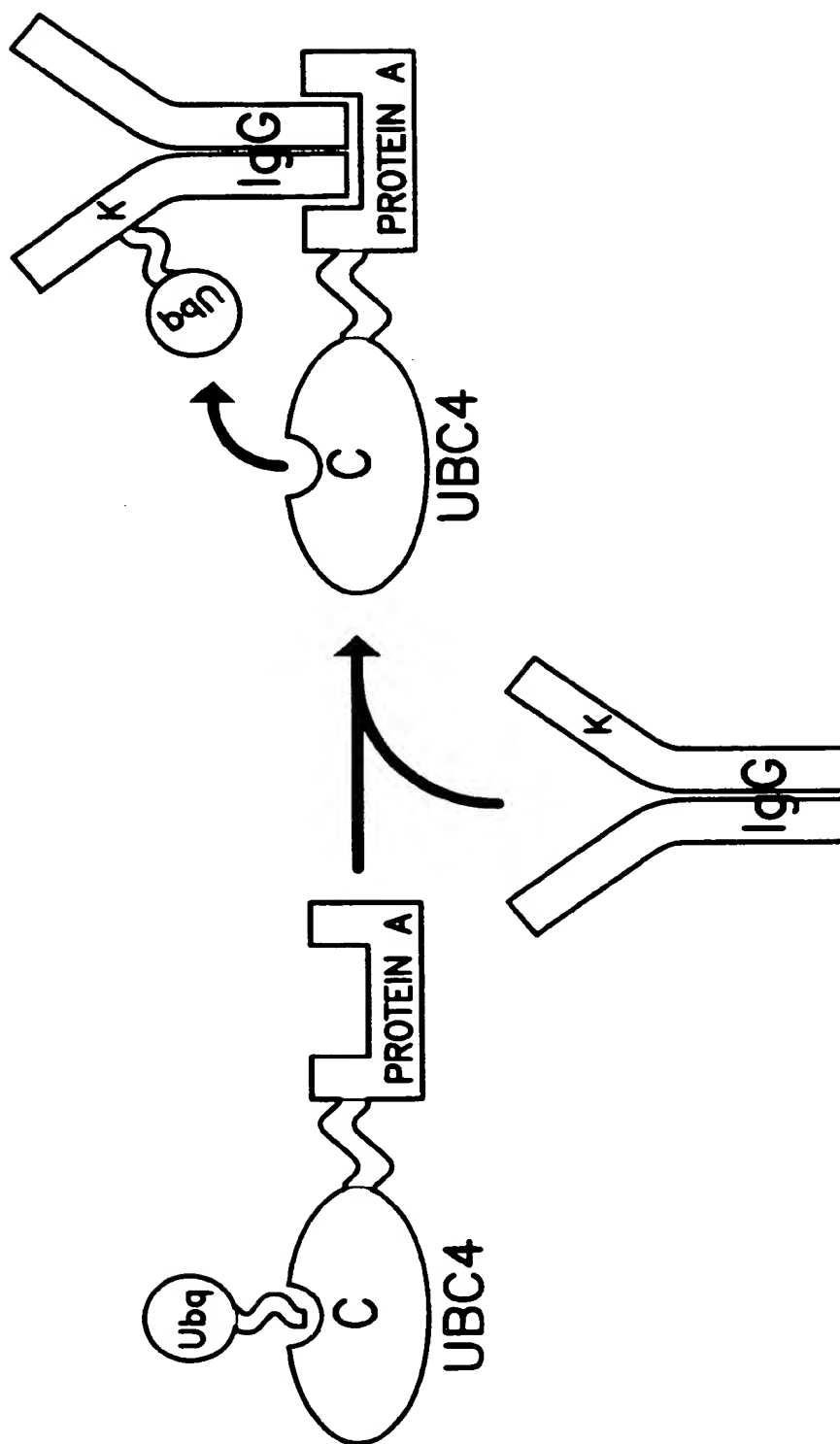


FIG. 6

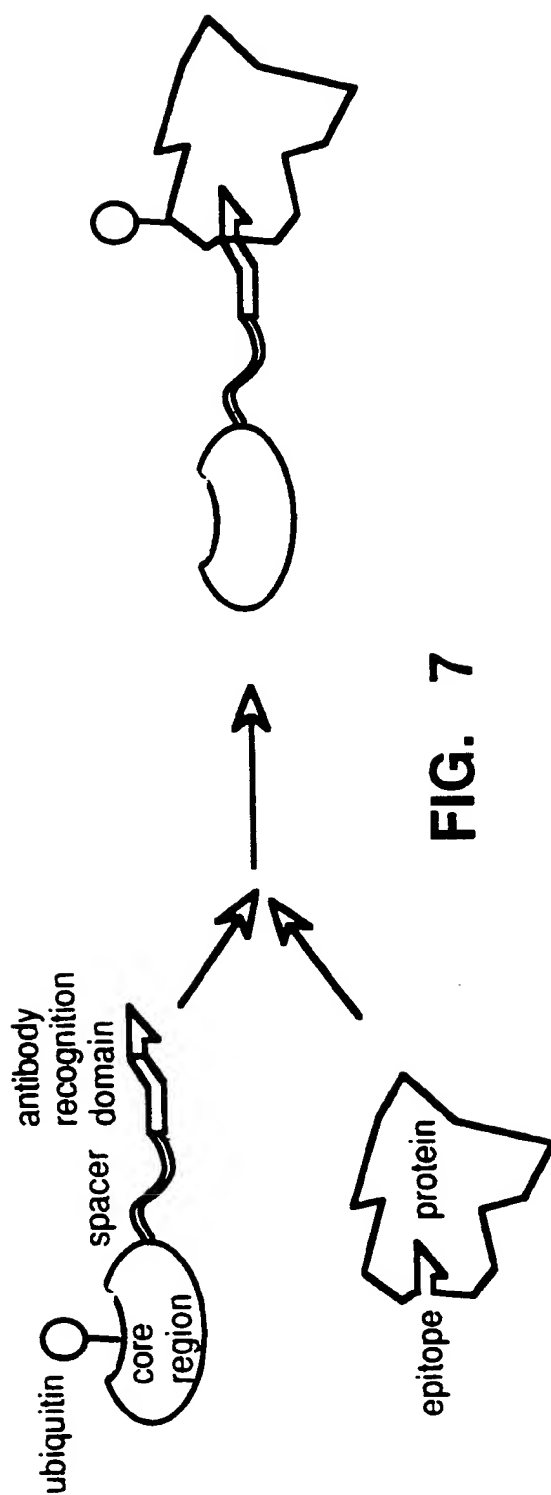
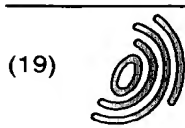


FIG. 7



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(11) **EP 0 626 450 A3**

(12) **EUROPEAN PATENT APPLICATION**

(88) Date of publication A3:  
01.05.1996 Bulletin 1996/18

(43) Date of publication A2:  
30.11.1994 Bulletin 1994/48

(21) Application number: **94303903.2**

(22) Date of filing: **31.05.1994**

(84) Designated Contracting States:  
**BE CH DE ES FR GB IE IT LI**

(30) Priority: **28.05.1993 US 70157**

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(54) **Ubiquitin conjugating enzyme (E2) fusion proteins**

(57) A novel class of fusion proteins based on the ubiquitin carrier protein, or E2, is described. The fusion proteins include, in addition to the E2 activity, a protein binding ligand having specific affinity for a target protein. It has been discovered that under cytosolic conditions, such E2 fusions will add a ubiquitin moiety to a target protein. Since ubiquitin addition triggers the endog-

enous cellular protein degradation pathway, such E2 fusion proteins can be used to selectively target proteins in a host for degradation. Thus, E2 fusion proteins genes can be introduced into transgenic organisms to defeat or inhibit natural activities or traits. The E2 fusion proteins can also be used by introduction into hosts for similar effects.

EP 0 626 450 A3



European Patent  
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# EUROPEAN SEARCH REPORT

Application Number  
EP 94 30 3903

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cls)
D,X	J. BIOL. CHEM., vol. 266, 1991 pages 23878-23885, M.L. SULLIVAN AND R.D. VIESTRA; 'Cloning of a 16-kDa ubiquitin carrier protein from wheat and Arabidopsis thaliana' *whole document*	1-3, 11-27	C12N15/62 C12N15/29 C12N1/21 A01H5/00 C12P21/00 C07K15/12 C12N9/10
X	J. BIOL. CHEM., vol. 267, 1992 pages 15116-15121, W.J. COOK ET AL.; 'Three-dimensional structure of a ubiquitin-conjugating enzyme (E2)' *abstract and discussion*	1-3, 11-27	
D,A	PROC. NATL. ACAD. SCI. USA, vol. 86, 1989 pages 9861-9865, M.L. SULLIVAN AND R.D. VIESTRA; 'A ubiquitin carrier protein from wheat germ is structurally and functionally similar to the yeast DNA repair enzyme encoded by RAD6' *abstract and Figure 5*	1-3	TECHNICAL FIELDS SEARCHED (Int.Cls)  C12N C12P C07K
A	TRENDS BIOCHEM. SCI., vol. 15, 1990 pages 195-198, S. JENTSCH ET AL.; 'Ubiquitin-conjugating enzymes: novel regulators of eukaryotic cells' *whole document*	1	
The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 8 February 1996	Examiner Yeats, S
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

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